PCT/AU03/00898

10/519890

29 DEC 2004

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Patent Office
Canberra

REC'D 31 JUL 2003
WIPO PCT

I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002950188 for a patent by THE UNIVERSITY OF ADELAIDE as filed on 12 July 2002.

COMMONWEALTH OF AUSTRALIA
PATENT OFFICE

WITNESS my hand this
Twenty-second day of July 2003

J. Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

Our Ref: 2402

P/00/009
Regulation 3.2

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED

Invention title: ALTERED INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

Name of Applicant: THE ADELAIDE UNIVERSITY

Address for Service: A.P.T. Patent and Trade Mark Attorneys
PO Box 222
Mitcham, S.A. 5062

The invention is described in the following statement:

FIELD OF THE INVENTION

This invention relates to an altered Insulin-like Growth Factor Binding Protein (IGFBP) with reduced IGF (Insulin-like Growth Factor) release characteristics. The IGFBP of the invention is proposed to be useful for therapeutic purposes, such as treatment of certain cancers. A specific form of the invention relates to an altered IGFBP-2.

BACKGROUND TO THE INVENTION

Insulin-like growth factors (IGF-I and IGF-II) are small, highly-related proteins (~7.5 kilodaltons) which mediate anabolic, mitogenic and anti-apoptotic activities in a wide variety of cell types. These actions result from IGF interaction with and subsequent activation of the type 1 IGF receptor (IGF1R) (Sepp-Lorenzino, (1998), Baserga, 1999). A second unrelated receptor (the type 2 IGF receptor or IGF2R) has the major function of regulation of IGF-II levels by internalisation and degradation (Wang *et al.*, 1994) and current evidence suggests that the IGF2R acts as a tumour suppressor of IGF-II-dependent tumours (Braulke, 1999).

IGFs are produced by the liver, providing circulating IGF, and are also secreted locally in most tissues. A family of 6 high-affinity IGF binding proteins (IGFBP-1 to -6) act to increase the half-life of IGFs in circulation (predominantly as the IGFBP-3·ALS·IGF complex) and also to transport IGFs to target tissues. Within target tissues IGFBPs can either enhance or inhibit IGF action. IGFBPs can inhibit the interaction of IGF by blocking binding to the IGF1R. However, under certain circumstances IGFBPs can release IGF, thereby making IGF available for binding to the IGF1R. This results in an enhancing effect on IGF action. . Release mechanisms include 1) proteolysis of the IGFBPs and 2) IGFBP binding to the extracellular matrix (ECM), both of which lower their affinity for IGF. Extracellular matrix binding is also believed to assist the localisation of IGF close to the cell surface and therefore near IGF1Rs. The outcome of IGFBP action is controlled by a balance between local proteolytic activity and the binding of IGFBPs to the ECM.

Substantial evidence (*in vivo* and *in vitro*) implicates insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) in cancer. Many tumour cells (including prostate and breast)

secrete more IGF-II and IGFBP-2 than their normal counterparts and their serum levels commonly rise as cancers progress (Cohen *et al.*, (1994); Thrasher *et al.*,(1996); Ho *et al.*, (1997); Chan *et al.*, 1998). IGF secreted by tumour cells binds to the Type 1 IGF receptor potentiating tumourigenesis and metastasis (DiGiovanni *et al.*, 2000).

The proteolysis of IGFBP-2 has been detected under a number of normal and abnormal physiological conditions. For example, IGFBP-2 fragments have been detected in human milk and cleavage occurred predominantly in the linker region between the N- and C-domains and including sites at residues 168 and 180/181 of hIGFBP-2 (Ho and Baxter, 1997; Elminger *et al.*, 1999). Proteolysed IGFBP-2 is also found in serum during pregnancy. IGFBP-2 is also cleaved by proteases produced by cancer cells (Michell *et al.*, 1997). The specific cancer cell proteases have not been well characterised although Cathepsin D produced *in vitro* by prostate epithelial cells has been shown to degrade IGFBP-2 (Kanety *et al.*, 1993; Nunn *et al.*, 1997). Preferential proteolysis of IGFBP-2 has been demonstrated in colonic cancers (Michell *et al.*, 1997) and neuroblastoma cells (Menouny *et al.*, 1997). Specific cleavage sites have not been described for proteolytic products generated by cancer proteases.

Protease cleavage sites have been identified in the IGFBP-3, -4 and –5 sequences. Proteolysis is generally within the linker regions of these proteins although it can be in the C-domain. Protease resistant IGFBP-4 and IGFBP-5 (Imai *et al.*, 1997) have been generated by mutating specific residues at cleavage sites or by deletion of some linker region residues (deletion of 121-141 of IGFBP-4 rendered it resistant to a protease in pregnancy serum (Byun *et al.*, 2000).

IGFBP-2 binds to human fibroblast extracellular membrane preparations (Arai *et al.*, 1996) and glycosaminoglycans (Russo *et al* 1997, Arai *et al.*, 1996). There are 2 potential matrix binding sites within the IGFBP-2 sequence. Current evidence suggests that the basic region of hIGFBP-2 (residues 227-244), corresponding to residues 201-218 of hIGFBP-5, may act as a site for matrix binding (Arai *et al.*, 1996). Using a synthetic peptide based on residues 201-218 of hIGFBP-5 (residues known to be important for matrix binding) Arai *et al.*, 1996, inhibited

IGFBP-2 binding to heparin-Sepharose. Hodgkinson *et al.*, (1994) predicted a glycosaminoglycan (GAG) binding site in IGFBP-2 based on a short GAG-binding consensus sequence described by Cardin and Weintraub (1989). This XBBXBX (B=basic, X=undefined) motif at residues 179-184 of hIGFBP-2 lies in the central domain. There is no published evidence that this motif plays a role in GAG binding.

SUMMARY OF THE INVENTION

This invention arises from the alteration of IGFBP-2 in a manner that inhibits the release of IGF-I and IGF-II. The inhibition of release results from the introduction of changes to the IGFBP-2 resulting in reduced binding to extracellular matrix (ECM) and reduced susceptibility to proteolysis by one or more proteases. This altered IGFBP-2 is proposed to be useful for its ability to inhibit growth of IGF-dependent tumours such as colon, prostate and breast cancers. This is to the inventor's knowledge the first time that an altered IGFBP has been constructed to contain both reduced ECM binding and reduced proteolytic susceptibility and the first time that this combination has been shown to be effective at inhibiting the release of IGF-I and IGF-II. It is proposed that this approach will be effective not only for IGFBP-2 but also for other IGFBPs. Additionally this is to the inventor's knowledge the first time that there have been functional data to show the location of the two ECM binding sites for IGFBP-2, and a demonstration that these together with alteration of proteolysis sites result in inhibition of the release of IGF-I and IGF-II. Additionally this is the first time to the inventor's knowledge that an overlapping IGF and ECM binding site has been altered in a manner that still allows IGF binding but not ECM binding.

The invention might in a first broad aspect be said therefore to reside in an altered IGFBP-2 molecule able to effect binding of IGF-I or IGF-II with high affinity characterised in an inhibited release of IGF on contact with extracellular matrix or exposure to a protease.

In a second form of the first aspect of the invention might be said to reside in an altered IGFBP molecule able to effect binding of IGF-I or IGF-II with high affinity characterised in an inhibited release of IGF on contact with extracellular matrix and exposure to a protease.

Cleaved IGFBP-2 has greater than 10 fold weaker affinity for IGF (Carrick, 2001). It is desirable for the altered IGFBP-2 to have at least to have an affinity for IGF-I equivalent to the IGF type 1 receptor which is 10 fold lower than that of native IGFBP-2 for IGF-I. This allows effective competition with the receptor for IGF binding and the term high affinity binding of IGF should be understood in that context.

Regions required for IGF binding have not been fully elucidated however considerable effort has been made to define these regions. Thus for example we (Hobba et al., 1998) and others (Zeslawski et al., 2001) have identified residues in the distal part of the N-domain involved in IGF binding. Similarly, the minimum C-domain length of IGFBP-2 required for IGF binding has been described (Forbes *et al.*, 1998). WO 00/23469 also describes the IGF binding domain which document is incorporated herein in its entirety by reference. Methods for assaying IGF binding are known in the art and can be readily employed to ascertain whether the altered IGFBP is able to bind IGF or not.

The inventor has identified two ECM sites for IGFBP-2 and shown that mutations in these lead to reduced heparin binding. The first ECM site for IGFBP-2 spans sequence 179-184 and consists of the sequence PKKLRP and was originally proposed on the basis of homology with the consensus sequence for matrix binding proteins (namely XBBXBX, Hodgkinson *et al.*, 1994). The second ECM binding sequence of IGFBP-2 identified is at 227-244 by homology with IGFBP-3 and IGFBP-5 and consists of sequence KHGLYNLKQCKMSLNGQR.

The invention might also in a third form of the first aspect be said to reside in an altered IGFBP-2 molecule able to effect binding of IGF-I or IGF-II with high affinity said IGFBP-2 molecule having alterations in the any one or more amino acids in both ECM binding sites, a

first ECM binding site being located at sequence 179 to 185 and a second ECM binding site being located at sequences 227 through 244, the alterations separately and together inhibiting the binding of the IGFBP-2 to ECM.

Preferably residues between 227 and 236 are substitution mutations because it is thought that at least part of this site may also be important for IGF binding. Whereas the 179-185 alteration may be achieved by deletion, inversion, substitution or other gross alteration, however preferably this is altered by amino acid substitution.

Generally substitution of a basic amino acid for an amino acid of different character, that is either neutral, or acidic is found generally to have a disrupting effect on matrix binding motifs.

It is found by the inventor that utilising the mutations so far introduced into IGFBP-2 that alteration of one of the two ECM sites alone is not sufficient to totally abolish ECM binding. Whilst the present invention preferably provides for the alteration of both ECM sites, the invention might also encompass the provision of alteration in one only of the ECM binding sites, and perhaps in combination with an altered protease cleavage and/or binding site.

Set out below are sequences shown or proposed to be the ECM binding sites for all six IGFBPs.

ECM binding sites (in the C-domain between the 14th and 16th cysteine residues)

IGFBP-3	CDKKGFYKKKQCRPSKGRKRGFC	(Firth, 1998)
IGFBP-5	CDRKGFYKRKQCKPSRGRKRGIC	(Arai, 1996b)
IGFBP-2	CDKHGLYNLKQCKMSLNGQRGEC	
	* * * *	
IGFBP1	CNKNGFYHSRQCETSMDGEAGLC	
IGFBP4	CDRNGNFHPKQCHPALDGQRGKC	
IGFBP6	CDHRGFYRKRQCRSSQGQRRGPC	

*=conserved positively charged residues

Sites for IGFBP 3 and IGFBP 5 have been published previously the present data establishes the binding site for IGFBP-2 and the sites for IGFBP1, 4, and 6 are suggested by reason of amino acid alignment.

The invention might also encompass an altered IGFBP-1,- 4, or -6 having an alteration in the ECM binding sites.

Alternatively in this first aspect of the invention it might be that a satisfactory decreased release of IGF might be achieved solely by inhibition of ECM binding by amino acid substitution at the second ECM binding site. Such decreased release might be the result of the close association between the IGF binding site and the second ECM binding site, and therefore the first aspect of the invention might reside in an altered IGFBP molecule and perhaps preferably an IGFBP-2 molecule that has one or more amino acid substitutions in the ECM binding site that still allows binding of IGF, whilst still inhibiting binding of ECM and thereby also inhibiting IGF release.

It is thought that the positively charged residues are important for binding and thus substitution of these are likely to result in inhibition of binding. The substitution might be a non conservative substitution such as the following alanine (A) substitutions for lysine (K).

Site 1	Site 2
PKKLRP	KHGLYNLKQCKMSLNGQR
PAKLRP	AHGLYNLKQCKMSLNGQR
PKALRP	KHGLYNLAQCKMSLNGQR
PKKLAP	KHGLYNLKQCAMSLNGQR
PAALAP	AHGLYNLAQCAMSLNGQR

Substitions of the lysine residues by other amino acids might also be contemplated.

The altered IGFBP-2 of the first aspect of the invention preferrably also has an alteration at and one or more proteolytic cleavage sites whereby to inhibit the release of an IGF when subjected to a protease specific for the proteolytic cleavage site.

A preferred form of the first aspect of the invention comprises substitution mutations at the first and second ECM binding sites, perhaps those referred to above, together with any one or more deletions in the linker domain that remove one or more proteolytic cleavage sites.

As indicated above in certain aspects the invention encompasses alterations that inhibit IGF release on exposure to one or more proteolytic enzymes. Many proteolytic enzymes to which IGFBPs are susceptible have as their target the linker domain. The proteolytic enzyme that the altered IGFBP is exposed to on delivery for therapeutic purposes may differ depending on the conditions to be treated. It is known that tumour cell lines vary in the proteolytic enzymes the produce. It has been shown by the inventor that a substantial deletion in the linker domain of IGFBP-2 leads to resistance to proteolytic cleavage whilst at the same time maintaining protein stability and maintaining IGF binding at high affinity. A preferred alteration of the IGFBP that leads to protease resistance is therefore one or more deletions within the linker domain. The size of the deletion or deletions may be varied. It has been found by the inventor that a deletion of substantially all of the linker region still led to IGF binding. With a deletion of substantially all of the linker domain it is preferred that amino acids from about 180 through to 191 are maintained.

Smaller deletions may also be suitable for removal of sites that make the IGFBP susceptible to proteolysis, thus deletions of amino acids about 110 through to about 170 has resulted in a decrease susceptibility to proteolysis.

Resistance to proteolysis may also be achieved by more targetted alteration to sites important for proteolytic cleavage or binding.

Substitution of residues other than those involved in proteolysis or ECM binding might also be contemplated, and these might be conservative substitutions or non-conservative substitutions, deletions, duplications, inversions and other rearrangements, provided that the altered IGFBP is still capable of high affinity IGF binding. Additionally the altered IGFBP might have additional changes such as glycosylations or other chemical modifications.

The proteins may be a fusion protein which assists in the purification, thus the protein may include a C terminal 6 histidine tag which provides for nickel affinity purification. Other fusion purification systems are also known and may be used. For bacterial systems a fusion protein may include a signal sequence, such as one derived from ompA, adapted to have the protein secreted through the surface of the cell.

In a second aspect the invention might be said to reside in a nucleic acid encoding one or more of the proteins or amino acid sequences of the first aspect of the invention. In a preferred form the nucleic acid is carried by a vector, the vector having nucleic acid operably linked with a control sequence including a promoter for transcription leading to expression of the protein or amino acid. Any one of the very many known vectors for that purpose may be used. Alternatively the vector may be used simply to introduce the nucleic acid encoding the altered IGFBP into a host cell for integration into a chromosome host cell, and might therefore be a retroviral expression vector.

In a third form the invention might be said to reside in a recombinant cell carrying a vector or the nucleic acid of the second aspect of the invention. The host cell might be intended for expression of the altered IGFBP which can thus be produced and purified such that the purified protein may be then separately used or administered as desired. The host cell might be bacterial, yeast, plant or mammalian. Alternatively the host cell may be intended for introduction into a treated animal such as a human for gene therapy purposes.

In a fourth form the invention might be said to reside in a pharmaceutical composition. The composition including the altered IGFBP-2 of the first aspect of the invention as an active

component. The pharmaceutical composition may be formulated in accordance with an approved method, and may include a carrier which may or may not be fused to the altered IGFBP, or conjugated with the altered IGFBP. The composition may additionally include other medicinal agents, pharmaceutical agents, adjuvants, diluent, excipients and the like.

The invention might in a fifth form also be said to reside in a method for decreasing serum and/or tissue levels of biologically active IGF-I or IGF-II by administering to a mammal an amount of altered IGFBP of the first aspect of this invention effective to inhibit the release of IGF-I or IGF-II from the altered IGFBP and thereby decrease serum and tissue levels of biologically active IGF-I or IGF-II.

The fifth aspect of the invention may contemplates using gene therapy for treating a mammal, using nucleic acid encoding the altered IGFBP. A nucleic acid sequence which encodes the altered IGFBP can be used for this purpose. The nucleic acid may injected directly into the patient, usually at the site where the altered IGFBP is required, perhaps as the site of a solid cancer. For *ex vivo* treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient.

Alternatively purified altered IGFBP can be administered to the mammal in a suitable carrier. the altered IGFBP may be administered orally parenterally, topically transdermally. It might be preferred to provide the altered IGFBP in slow release from. Determination of appropriate dosages and formulation may be achieve by one of ordinary skill in the art using only routine experimentation. See for example Remington's Pharmaceutical Sciences (Martin E.E. ed, latest edition), Mack Publishing Co, Easton PA.

Conditions that might be amenable to treatment in this way include cancers of the breast , prostate, colorectal, lung, thyroid, ovaries, and brain as well as in childhood leukaemias, glioblastomas, and neuroblastomas.

It is contemplated that treatment of say prostate cancer according to the present invention may not be used on its own but as an adjunct to other methods.

By way of a shorthand notation the following three and one letter abbreviations for amino acid residues are used in the specification as defined in Table 1.

Where a specific amino acid residue is referred to by its position in the polypeptide of an protein, the amino acid abbreviation is used with the residue number given in superscript (i.e. Xaan)

TABLE 1

Amino Acid	Three-letter Abbreviation	One letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P

Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic diagram of the hIGFBP-2 expression vector constructed from the mammalian expression vector pXMT2 (Rathjen *et al.*, (1990); Whyatt *et al.*, (1993)). A colicin E1 origin of replication (ori) and the b-lactamase ampicillin resistance gene (Amp) enable propagation in E. *coli*. Mammalian expression of the IGFBP-2 cDNA is driven by the adenovirus major late promoter (MLP). The dihydrofolate reductase (DHFR) gene is present for selection. A 6 Histidine tag is encoded at the 3' end of the IGFBP-2 cDNA sequence.

Figure 2. Purified hIGFBP-2 and mutants were separated on a 12% tricine SDS polyacrylamide gel. Proteins were stained with Coomassie blue (except for Des(114-170)His hIGFBP-2 which was stained with sypro ruby). All mutants migrated with the expected size as estimated by comparison with molecular size standards (Novex Broad range). A minor breakdown product was detected in this particular K234AHis preparation.

Figure 3. Schematic of hIGFBP-2 and truncated hIGFBP-2 showing the conserved N- and C-cysteine (=stripe) domains connected by the linker domain. The know disulphide bonds and residue numbers are indicated above. Potential cleavage and ECM sites are also identified.

Figure 4. BIAcore analysis of hIGFBP-2 and mutants binding to IGF-I. Human IGFBP-2 (at concentrations of 3.1, 6.25, 12.5, 25 and 50nM) was allowed to associate with the Biosensor surface (70 resonance units of hIGFBP-2) for 300s (from t=150s) and then dissociated for 900s. Real time binding is measured in response units. Kinetic studies were performed at a flow rate of 30 µl/min to minimize mass transfer effects. The IGF-I-coated biosensor surface was regenerated with 10mM HCl between binding cycles. For each binding curve the response obtained using control surfaces (no protein coupled) was subtracted.

Table 1. Kinetic constants obtained from BIAcore analysis of hIGFBP-2 and mutant hIGFBP-2 binding to IGF-I. Data was analysed using BIAevaluation software 3.0 and fit to a Langmuir 1:1 binding model. The dissociation constant (K_D) was determined from the calculation of k_d/k_a, where k_a is the association rate and k_d is the dissociation rate. Relative K_D is equal to K_D of hIGFBP-2/K_D of hIGFBP-2 mutant.

Table 2. Kinetic constants obtained from BIAcore analysis of hIGFBP-2 and mutant hIGFBP-2 binding to IGF-II. Data was analysed using BIAevaluation software 3.0 and fit to a Langmuir 1:1 binding model. The dissociation constant (K_D) was determined from the calculation of k_d/k_a, where k_a is the association rate and k_d is the dissociation rate. Relative K_D is equal to K_D of hIGFBP-2/K_D of hIGFBP-2 mutant.

Figure 5. List of cell lines used as sources of conditioned media containing proteolytic activity. Cell lines are grouped in to cancer types. A qualitative score is given to each cell line indicating the amount of proteolytic activity evident upon incubation with hIGFBP-2 (central column). If the truncation of hIGFBP-2 (Des(114-170)His hIGFBP-2)

resulted in protection against cleavage by conditioned media compared with hIGFBP-2 then this is indicated in the column on the right.

Figures 6 and 7. Analysis of susceptibility of truncated hIGFBP-2 to proteolysis in culture media conditioned by cancer cells. Truncated hIGFBP-2 and hIGFBP-2 were exposed to culture media of a) T84 colon cancer, b) PC3 prostate cancer, c) DU145 and d) LNCaP prostate cancer cell lines for 0, 3 or 24 hours at 37 degrees. Samples were separated by SDS polyacrylamide gel electrophoresis on 10% tricine gels and transferred to nitrocellulose. hIGFBP-2 and cleavage products were detected with an anti-IGFBP-2 polyclonal antibody (left). hIGFBP-2 migrates at 34kDa whereas Des(114-170)His migrates at 20kDa. A dimer is present in hIGFBP-2 preparations. Proteolytic fragments range in size between 14 to 20 kDa. Densities of bands indicated on the blots were quantitated using the NIH image program and amounts of uncleaved or cleaved hIGFBP-2 graphed (right).

Figure 8. Heparin binding affinities of hIGFBP-2 and K234AHis IGFBP-2 were measured by surface plasmon resonance. Data was analysed using BIAevaluation software 3.0 and fit to a Langmuir 1:1 binding model. The dissociation constant (K_D) was determined from the calculation of k_d/k_a, where k_a is the association rate and k_d is the dissociation rate.

DETAILED DESCRIPTION OF THE INVENTION

Materials and Methods

Mutagenesis and subcloning

Mutations of cDNA encoding hIGFBP-2 in the pBluescript vector (Stratagene, La Jolla, CA, USA) were introduced using the Quikchange mutagenesis method (Stratagene). The following oligonucleotides were used to introduce the lysine (K) to alanine (A) mutations and the deletion mutant Des(114-170)His:

K180A K181AHis	Forward	5'CTT GGC CTG GAG GAG CCT GCC GCC CTG CGA CCA CCC CCT 3'
	Reverse	5'AGG GGG TGG TCG CAG GGC GGC AGG CTC CTC CAG GCC AAG 3'
K227AHis	Forward	5'ATC CCC AAC TGT GAC GCC CAT GGC CTG TAC ACC 3'
	Reverse	5'GGT GTA CAG GCC ATG GGC GTC ACA GTT GGG GAT 3'
K234AHis	Forward	5'GGC CTG TAC AAC CTC GCC CAG TGC AAG ATG TCT 3'
	Reverse	5'AGA CAT CTT GCA CTG GGC GAG GTT GTA CAG GCC 3'
K237AHis	Forward	5'AAC CTC AAA CAG GCC ATG TCT CTG AAC GGG 3'
	Reverse	5'CCC GTT CAG AGA CAT GGC GCA CTG TTT GAG GTT 3'
Des(114-170)His	Forward1	5'GTT GCA GAC AAT GGC GCC GGC CAC TCA GAA GAA GCC 3'
	Reverse1	5'GCC TCC TTC TGA GTG GCC GGC GCC ATT GTC TGC AAC 3'
	Forward2	5'CGG CAC ATG GGC AAG GCC GGC AAG CAT CAC CTT 3'
	Reverse2	5'AAG GTG ATG CTT GCC GGC CTT GCC CAT GTG CCG 3'

The deletion mutant Des(114-170)His was generated by sequentially introducing two NaeI restriction sites in the cDNA encoding residues 114 and 170 respectively. The new clone was then digested with NaeI and religated to delete out the sequence between these sites.

The cDNA clones encoding resultant mutant IGFBPs were transformed into DH5α E. *coli*. Clones were sequenced to confirm correct introduction of mutations. They were subsequently subcloned into the pXMT-2 vector using XhoI and EcoRI restriction sites and transformed into DH5α E. *coli* (see Fig 1; Rathjen P.D. *et al.*, (1990); Whyatt L.M. *et al.*, (1993)). Mutant IGFBP-2 proteins were expressed upon transient transfection of COS-1 monkey kidney cells (ATCC:CRL 1650) with the mutant IGFBP-2 cDNAs.

Purification and Analysis of purity

Proteins were purified using standard Nickel affinity purification techniques taking advantage of a 6 histidine tag at the C-terminus of each protein. Purification is from culture medium as the IGFBP is secreted (Forbes et al, 1998). Following elution from the nickel column, proteins were further purified using reverse phase high performance liquid chromatography (HPLC). Purity was analysed by rpHPLC, SDS PAGE and mass spectrometry. The mass of each mutant was determined by electrospray mass spectrometry (determined by Dr. Chris Bagley, Hanson Centre) and found to be correct (generally within the limits of the mass spec=1 mass unit/10,000 daltons).

Measurement of IGF binding affinities

IGF binding affinities of hIGFBP-2 and mutants were determined by surface plasmon resonance using the BIAcore with IGF-I or IGF-II coupled to the sensor surface (for details of method see Carrick *et al.*, (2001). IGF-I or IGF-II (70RU) was coupled to a CM-5 biosensor chip (BIAcore Inc) via amine groups using standard coupling procedures (Lofas and Johnsson, 1990). Briefly, at 5µl/min a CM5 chip was activated with 35µl NHS (0.4mg)/EDC (2.6mg) and then 35µl IGF (10µg/ml) was coupled in 10mM sodium acetate pH 4.5. Unreacted groups were inactivated with 35µl 1M ethanolamine-HCl, pH 8.5. Kinetic studies with a range of hIGFBP-2 or mutant concentrations (50, 25, 12.5, 6.25 and 3.1nM) were performed at 40µl/min to minimize mass transport effects with 300 secs allowed for association and 900 secs for dissociation. The IGF surface was regenerated with 10mM HCl.

Proteolysis Assay

The source of proteases for the proteolysis assays was conditioned medium of cancer cells. Cells were grown to confluence in the presence of foetal calf serum. (T84 cells are grown in DMEM: Ham's F12 (50:50 v:v) with 10% foetal bovine serum FBS; LNCaP were grown in RPMI+6%FBS; PC3 and DU145 were grown in DMEM+10%FBS, all media and FBS are from GIBCO). Cells were then washed 2x2 hours in serum free culture medium. Cells were then cultured for 3 days in serum free conditions and the medium was collected. Conditioned medium was concentrated approximately 10 fold using a centricon-10 (Millipore Corp, MA

USA). Purified hIGFBP-2 or mutants (250ng in 2µl) were mixed with conditioned medium for 24 hours at 37°C to allow proteolysis. Proteins were separated on 12% tricine SDS polyacrylamide gels and transferred to nitrocellulose. Nitrocellulose filters were probed with a specific polyclonal anti-IGFBP-2 antibody (raised in our laboratory) to detect IGFBP-2 and IGFBP-2 fragments. A secondary goat anti-rabbit antibody conjugated to avidin alkaline phosphatase (Sigma) was used to detect the anti-IGFBP-2 antibody. Substrates for avidin alkaline phosphatase (nitroblue tetrazolium and 5 bromo 4 chloro 3-indolyl phosphate p-toluidine salt) were added and coloured bands indicated presence of IGFBP-2.

Matrix binding assay

Heparin was biotinylated using biocytin hydrazine (Pierce) using conditions recommended by the manufacturer. Following the reaction the biotinylated heparin was concentrated using a centricon-3 (Millipore Corp, MA USA) and was dialysed against H_20. Biotinylated heparin was coupled to a streptavidin biosensor chip in 0.3M NaCl and HBS (hepes buffered saline containing surfactant, BIAcore Inc.). hIGFBP-2 and mutants at different concentrations (6.25nM to 300nM) were injected at 10µl/min. Regeneration of the surface was achieved with 2M NaCl.

Results and Discussion

Cloning, expression, purity and IGF binding affinities

Five mutants have been designed for introduction of protease resistance or interruption of matrix binding (K180A K181AHis, K227AHis, K234AHis, K237AHis, Des(114-170)His). These were purified to homogeneity (Figure 2) and subjected to mass spectral analysis to confirm they had the expected mass. The residues K180 and K181 are potential sites of protease cleavage (Ho, J.P. & Baxter, R.C. (1997) and are also possibly involved in matrix binding (Hodgkinson, et al (1994)). K227, K234 and K237 are residues in the analogous regions of IGFBP-3 and –5corresponding to matrix binding motifs. The potential sites of proteolytic cleavage and matrix binding are highlighted in Figure 3.

The purified mutants were tested for their ability to bind IGF-I by BIAcore analysis (Figure 4). All mutants have similar affinities to native hIGFBP-2. Mutant K237AHis has about 2 fold higher affinity for both IGF-I and IGF-II and Des(114-170)His has a 5 fold or 2 fold decrease in affinity for IGF-I and IGF-II respectively (Tables 1 and 2).

Protease Assays

The mutant binding proteins were tested for protease sensitivity in the assay described in materials and methods. We first analysed the truncation mutant Des(114-170)His and observed protease resistance in a number of conditioned media including T84, HT29, CaCO (all colon cancer cells) and PC3 (prostate cancer cell line). Figure 5 outlines which cell lines were used, the relative amount of protease activity (qualitative from observations of gels) and which conditioned media contained proteases which are no longer able to cleave the truncation mutant.

Importantly, there was a significant amount of easily detectable protease activity in the T84 cell line conditioned medium (Fig 6 and 7). The truncation mutant was clearly resistant to proteolysis in this medium. Proteolysis of hIGFBP-2 was also easily detected in PC3 conditioned medium and the truncation mutant was also resistant to proteolysis by this medium(Fig 6). In other cell lines there was less proteolytic activity making detection of protease resistance more difficult (eg LIM1215). In other cell lines the truncation mutant was clearly proteolysed (Fig 7).

These results highlight the fact that each cell line produces a different array of proteases. It is not known which protease is cleaving hIGFBP-2 in the T84 and PC3 media. The cleavage product in T84 medium corresponds to a C-terminal fragment of hIGFBP-2 as detected by an antibody specific for C-terminal residues. We have tested the other mutants in the T84 conditioned medium for protease resistance. As these residues lie outside the 114-170 truncation it was not surprising that none were resistant to proteolysis, suggesting that cleavage does not occur at K180, K181, K227, K234, K237.

Heparin binding

We analyzed matrix binding using the commonly used model system of heparin binding. We analyzed heparin binding using the BIAcore. Preliminary data show that the K234A mutation reduces heparin binding 5 fold (Fig 8) and the K180A, K181A mutation has a great effect on heparin binding. This data indicates that there are probably 2 heparin binding sites on IGFBP-2.

REFERENCES

Arai *et al.*,. *Endcrinology* **137**, 4571-4575 (1996)

Arai *et al., J. Biol. Chem.* **269**, 20388-20393 (1996)

Baserga, *Exp. Cell Res.* **253(1),**1-6 (1999)

Braulke, *Horm. Metab. Res.* **31(2-3),** 242-246 (1999)

Byun *et al., J Clin Endocrinol Metab* 85(1):373-81 (2000).

Chan *et al., Science* **279**, 563-566 (1998)

Cardin. & Weintraub *Arteriosclerosis* **9**, 21-32 (1989).

Carrick *et al., J. Biol. Chem.* **276,** 27120-27128 (2001)

Cohen *et al., J. Clin. Endocrinol. Metab.* **79,** 1410-1415(1994)

DiGiovanni *et al., PNAS* **97,** 3455-3460 (2000)

Elminger *et al., Mol. Cell. Endocrinol.* **175(1-2),** 211-218 (1999)

Firth *et al., J. Biol. Chem.* **273**, 2631-2638 (1998)

Forbes *et al., J. Biol. Chem.* 273, 4647-4652 (1998)

Ho & Baxter, *Clin. Endocrinol.* 46, 333-342 (1997)

Ho & Baxter *Endocrinology* **138**, 3811-3818 (1997).

Hobba *et al., J. Biol. Chem.* **273**:19691-19698 (1998).

Hodgkinson *et al. J. Mol. Endocrinol.* **13**, 105-112 (1994);

Imai *et al., J. Clin. Invest.* 100(10), 2596-2605 (1997)

Kanety, *et al. J. Clin. Endocrinol. Metab.* **77,** 229-233 (1993).

Lofas and Johnsson, *J. Chem. Soc. Chem. Commun.* **21,** 1526-1528 (1990)

Menouny *et al., Endocrinology* **138**, 683-690 (1997).

Michell *et al., Br. J. Cancer* **76**, 60-66 (1997).

Nunn *et al.*, *J. Cell. Physiol.* **171**, 196-204 (1997).

Rathjen *et al.*, *Cell* **62(6)**,1105-1114 (1990)

Russo *et al. Endocrinology* **138**, 4858-4867 (1997)

Sepp-Lorenzino, *Breast Cancer Res. Treat.* **47(3)**, 235-253 (1998)

Thrasher *et al.*, *J. Urol.* **155**, 999-1003 (1996)

Wang *et al.*, *Nature* **372**, 464-467 (1994)

Whyatt *et al.*, *Mol. Cell. Biol.* **13**, 7971-7976 (1993)

Zeslawski *et al.*, *EMBO J.* **20**, 3638-44 (2001)

Dated this 12 day of July 2002

THE ADELAIDE UNIVERSITY
By their Patent Attorneys
A.P.T. Patent and Trade Mark Attorneys

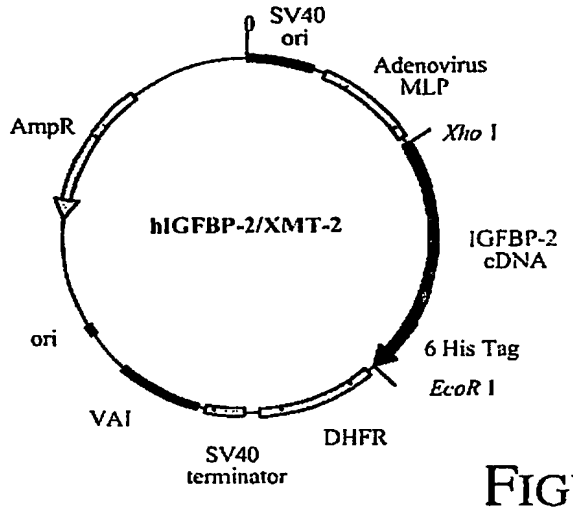


FIGURE 1

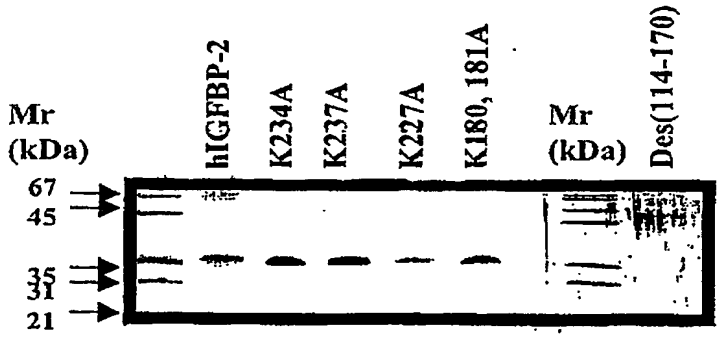
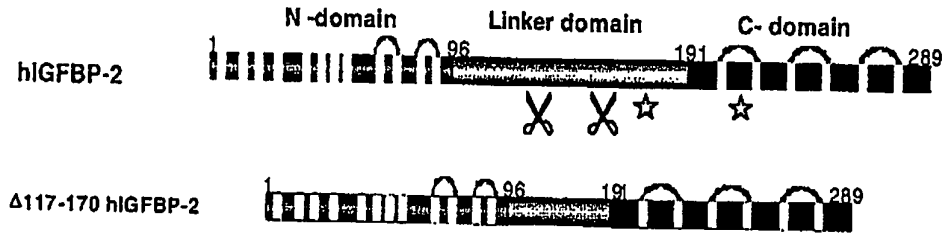


FIGURE 2



Schematic of humanIGFBP-2 and truncated hIGFBP-2 sequence showing the N- and C-terminal cysteine (=stripe) rich domains separated by the central domain. The known disulphide bonds and residue numbers are indicated above.
✂ =possible cleavage sites
☆ =possible matrix binding sites

FIGURE 3

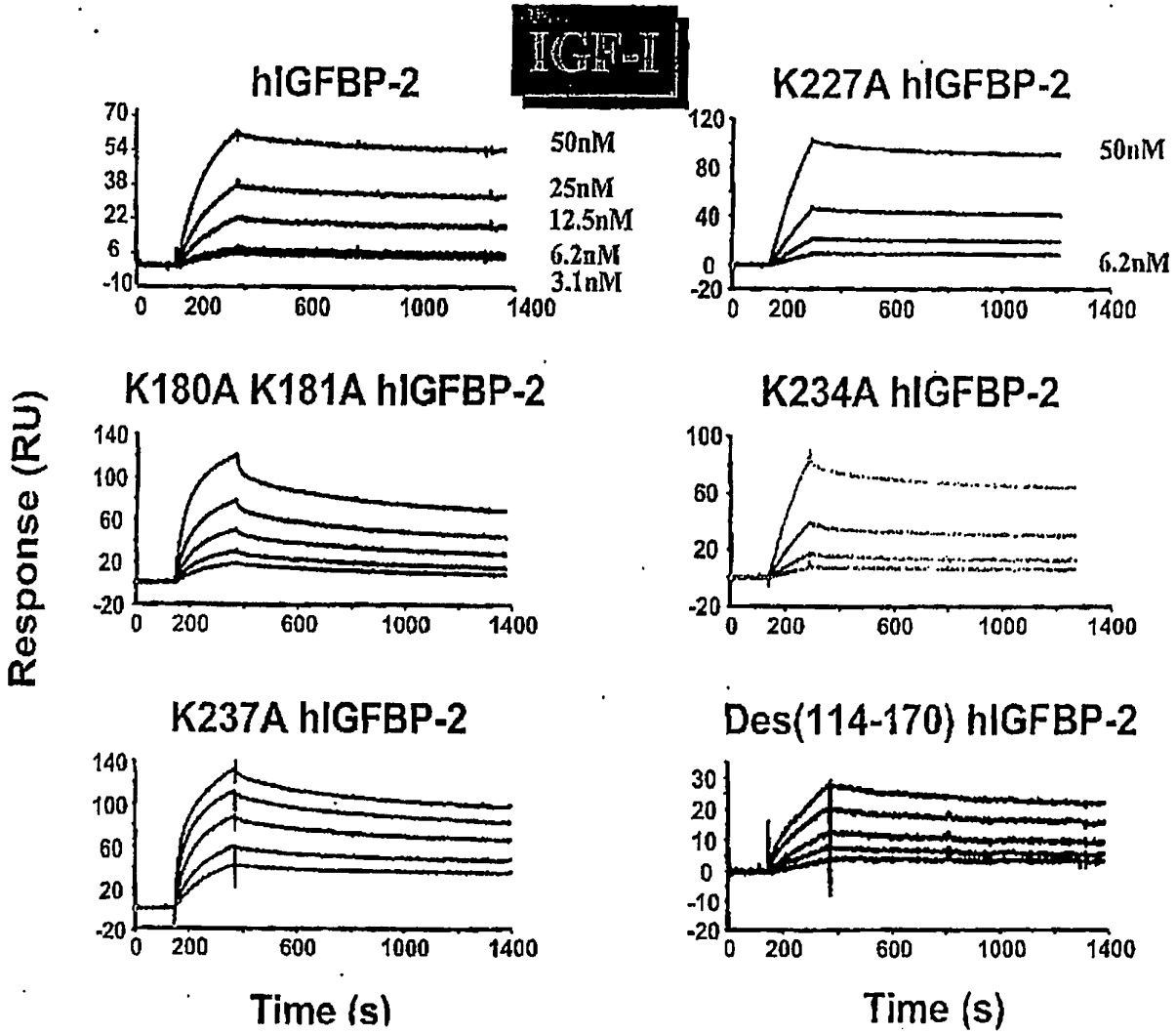

IGF-I
hIGFBP-2
K227A hIGFBP-2
K180A K181A hIGFBP-2
K234A hIGFBP-2
K237A hIGFBP-2
Des(114-170) hIGFBP-2
50nM
25nM
12.5nM
6.2nM
3.1nM
50nM
6.2nM
Response (RU)
Time (s)
Time (s)

FIGURE 4

	k_a x10^5 (1/Ms)	k_d x10^{-4} (1/s)	K_D x10^{-10} (M)	Rel. K_D
hIGFBP-2	1.8	1.74	9.67	1.0
K180A K181A	6.41	6.8	11.1	0.87
K227A	1.91	1.37	7.17	1.34
K234A	1.69	2.52	14.9	0.64
K237A	7.24	3.06	4.22	2.29
Des(114-170)	1.17	4.25	36.3	0.26

TABLE 1

	k_a x10^5 (1/Ms)	k_d x10^{-4} (1/s)	K_D x10^{-10} (M)	Rel. K_D
hIGFBP-2	2.38	1.4	5.89	1.0
K180A K181A	6.51	5.58	8.57	0.68
K227A	2.09	0.96	4.61	1.27
K234A	1.81	2.86	15.8	0.37
K237A	7.24	1.23	2.01	2.9
Des(114-170)	1.14	1.3	11.4	0.51

TABLE 2

	Proteolytic Activity	Protection
Prostate Cancer		
LNCaP	★★★	No
PC3	★	Yes
DU145	★★★	No
Colon Cancer		
HT29	★	Yes
SW480	★★	No
LIM1215	★★	Not sure
CaCo	★	Yes
T84	★★★	Yes
Breast Cancer		
MCF7	★	No

FIGURE 5

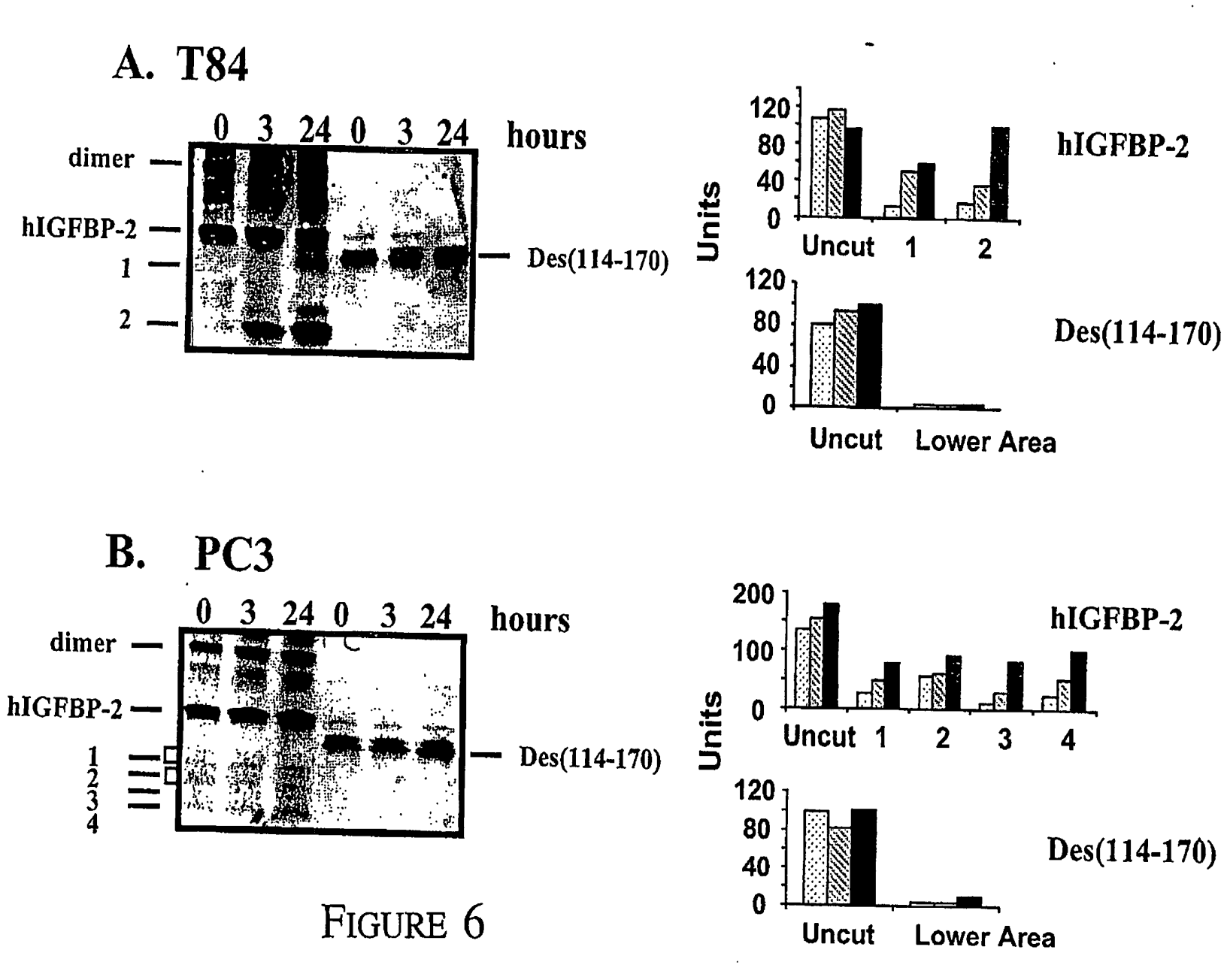
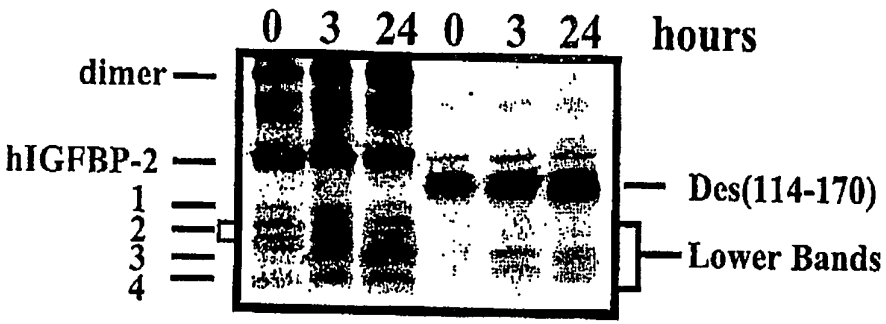
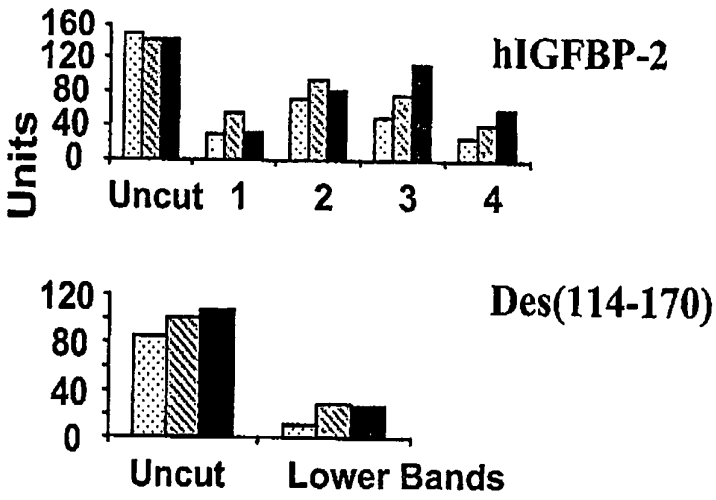

A. T84
0 3 24 0 3 24
hours
dimer
hIGFBP-2
1
2
Des(114-170)
Units
120
80
40
0
Uncut 1 2
hIGFBP-2
120
80
40
0
Uncut Lower Area
Des(114-170)
B. PC3
0 3 24 0 3 24
hours
dimer
hIGFBP-2
1
2
3
4
Des(114-170)
Units
200
100
0
Uncut 1 2 3 4
hIGFBP-2
120
80
40
0
Uncut Lower Area
Des(114-170)
0 hr
3hr
24hr

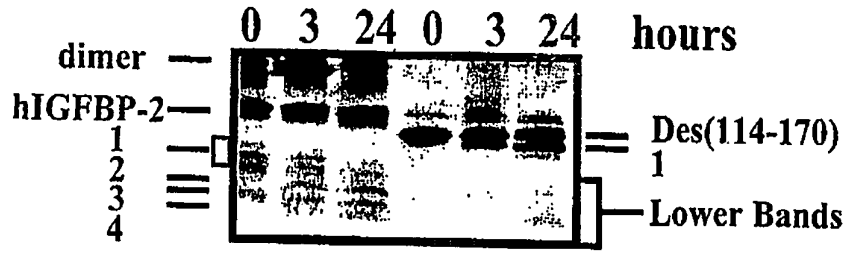
FIGURE 6

C. DU145





D. LNCaP



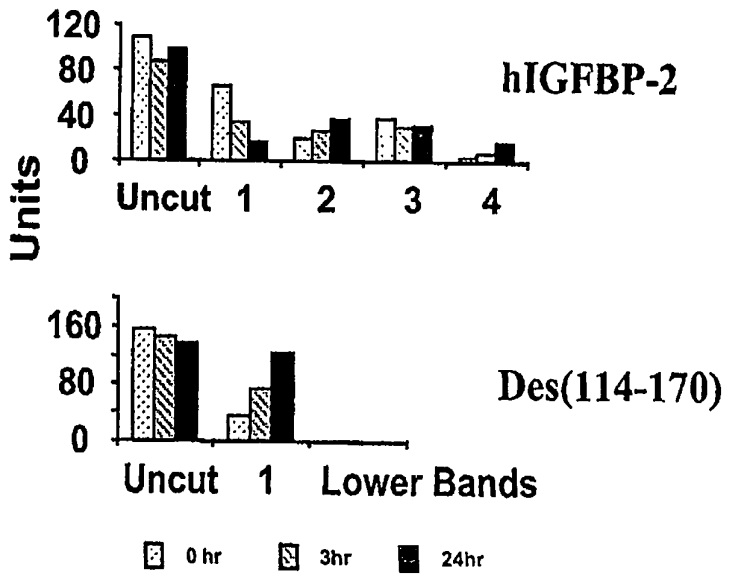


FIGURE 7

Heparin binding by hIGFBP-2 and mutants

	K_D (M)
hIGFBP-2	$1x10^{-8}$
K234A hIGFBP-2	$4.82x10^{-8}$

FIGURE 8